FREEZE-DRYING OF BIOLOGICAL SPECIMENS FOR ELECTRON MICROSCOPY USING THERMOELECTRIC COOLING

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Freeze-drying for electron microscopy is one of the most successful methods for avoiding specimen distortion caused by drying of liquid solutions in air. It has been calculated that the surface forces of receding droplets subject small specimens to pressures of the order of tons per square inch (1). In freeze-drying, an aqueous solution of the specimen is rapidly frozen and the ice allowed to sublime under vacuum. Thus, the specimen is maintained in a rigid state and the surface tension forces are greatly reduced. Several studies have demonstrated a well preserved three-dimensional structure in frozen dried material, as compared to the same material dried in air (2–5).

However, freeze-drying has not been widely used, largely because of inherent difficulties associated with existing methods which necessitate the use of separate vacuum systems, special glassware, and glass-to-metal vacuum seals. Relatively large areas of the apparatuses become cold and act as water vapor traps for the atmosphere so that several hours are often required for complete sublimation. Most methods and equipment used to date involve transfer of the frozen dried specimen from the freeze-drying apparatus through the atmosphere to a vacuum evaporator for replication and/or heavy metal shadowing. Even if the specimen is warmed before transfer, absorption of water and consequent distortion of the specimen may occur.

Recently there have been developed cooling devices that employ semiconductor materials to amplify the Peltier thermocouple effect. In these devices, a direct current is passed through the junction of two dissimilar conductors (usually copper and bismuth). Depending upon the direction of current flow, heat is either absorbed or generated. The freeze-drying apparatus described here consists simply of a commercially available thermoelectric unit placed within a standard vacuum evaporation unit.

The thermoelectric device has several advantages, among which are the following: only electrical connections are required for its operation, eliminating the need for liquid nitrogen trapping and freezing; the volume of frozen material is

small, so that only a few minutes are required for complete sublimation; sublimation, shadowing, and/or replication can all be performed in the same vacuum, eliminating the possibility of condensation and surface distortion during transfer through the atmosphere; the temperature can be electrically controlled throughout the process; air-dried controls can be prepared simultaneously.

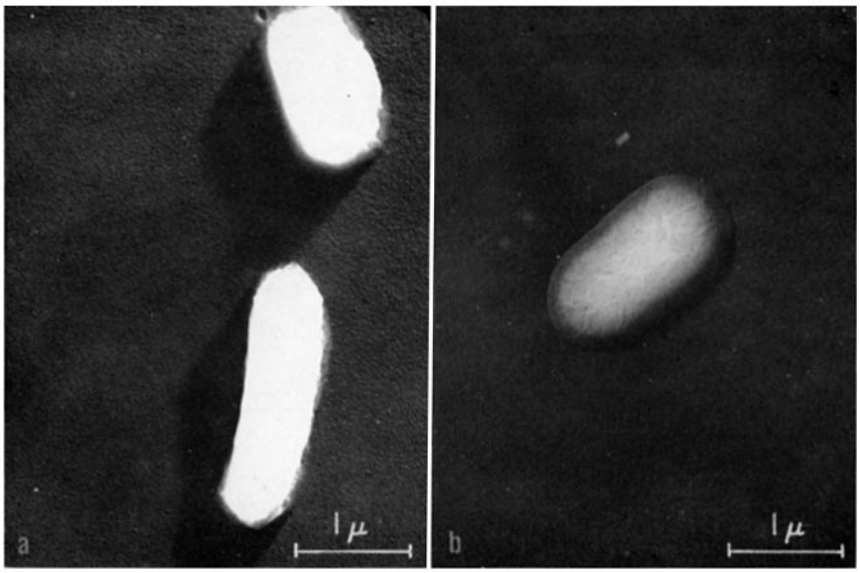

FIGURE 1 *Escherichia coli:* uranium-shadowed; (*a*) frozen-dried; (*b*) air-dried. The air-dried particle is much flatter than the frozen-dried, as evidenced by the shadow lengths, even though the shadow angle was the same for both preparations. × 15,000.

MATERIALS AND METHODS

Two different thermoelectric devices were used. The first, a commercial model TA-20M thermomodule, manufactured by Ohio Semiconductors, Columbus, Ohio, requires a current of 30 amp d-c at 3 to 4 v for optimum performance. The second device, constructed for us by Hughes Aircraft Co., Culver City, California (Hughes reference 61588), requires 25 amp d-c at only 0.1 to 0.2 v. Both units are about 2½ inches square by 1 inch high, and require d-c power supplies with less than 10 per cent ripple. The power supply used with the Ohio device was manufactured by General Electric and supplied by Ohio Semiconductors; that used with the Hughes unit was constructed by us.

The stainless steel base plate of the Kinney SC-3 vacuum evaporator, which was used in this work, is not a good enough heat conductor to dissipate, without special cooling, the 90 to 120 w generated by the Ohio device. To overcome this difficulty, a base plate was made from ½ inch flat stock aluminum, in which holes were drilled to accommodate the evaporator electrodes and ports. A neoprene gasket was used as a vacuum seal between the aluminum and stainless steel base plates. The hot side of the cooling device was bolted to the aluminum plate and was thermally connected with a thin layer of vacuum grease. Because of its lower power characteristics, the Hughes device could be bolted directly to the stainless steel plate of the SC-3 evaporator. The two leads of the thermoelectric units were connected to two of the electrodes within the evaporator. The external parts of the electrodes were connected to the power supply.

The temperature of the flat cold plate of the thermoelectric units was measured with an iron-constantan thermocouple. Two evaporator electrodes provided electrical connections for the thermocouple junctions. Both devices reached temperatures of −30° to −35°C in air at 18° to 23°C. Under vacuum, the Hughes device reached −65°C and the Ohio unit −45°C.

The frozen-dried specimens illustrated here were prepared in one of the following manners: the optimum current was passed through the thermoelectric unit, which attained minimum tempera-

ture within a few minutes. If the cold plate was to be exposed to the atmosphere for more than a few minutes, it was covered with a small beaker to prevent excess condensation. A thin, freshly cleaved piece of mica about 2 cm square was placed on the cold plate and held down with small weights. An aqueous suspension of the specimen was either sprayed or dropped on the mica and ticles adhered to the films, so that they were, in reality, pseudoreplicas.

It was found that silicon monoxide films were not disrupted by the temperature changes in the freeze-drying process, so that an alternate preparation method was developed in which nickel electron microscope grids coated with thin SiO films were placed directly on the cold plate surface

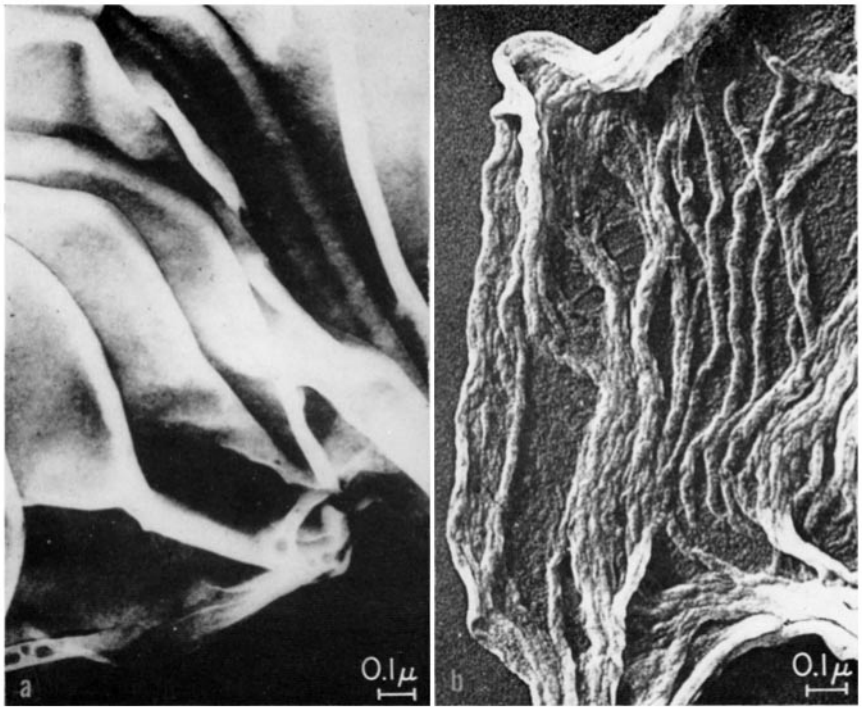

FIGURE 2 Marginal bands of erythrocytes of the lizard *Dipsosaurus dorsalis*: platinum-shadowed, carbon-replicated; (*a*) frozen-dried; (*b*) air-dried. The diameters of the frozen-dried bands (400 to 500 A) are about twice those of the air-dried bands. × 50,000.

was rapidly frozen. The bell jar was placed over the base plate and evacuation started. The ice film on the mica disappeared during the rough pumping cycle, but as routine we kept the specimen at about −30°C for about 15 minutes, or until a high vacuum was attained. Dry ice was placed in the cold trap of the evaporator diffusion pump in order to condense any volatile material that might otherwise be deposited on the specimen. After a sufficient vacuum was attained, the cold plate was warmed to ambient temperature, and the specimen was shadowed with platinum and replicated with carbon. The carbon-platinum films were floated off on the surface of water and picked up on microscope grids. In most cases the specimen particles adhered to the films, so that they were, in reality, pseudoreplicas.

It was found that silicon monoxide films were not disrupted by the temperature changes in the freeze-drying process, so that an alternate preparation method was developed in which nickel electron microscope grids coated with thin SiO films were placed directly on the cold plate surface which had been lightly coated with vacuum grease. The plate was cooled to −35°C, and the specimen was sprayed directly on the grids. Sublimation was carried out as described above, and the grids were shadowed with uranium. This method has the advantage that the grids are immediately ready for electron microscope observation, and neither replication nor film transfer is necessary. Little difference was observed among specimens prepared by both methods.

RESULTS AND DISCUSSION

The specimens described here were suspended in distilled water or 0.001 M ammonium acetate before freeze-drying. Figs. 1 *a* and 1 *b* are electron

micrographs of *Escherichia coli*, frozen- and air-dried, respectively. Both specimens were shadowed at the same time and at approximately the same shadow angle. It is apparent from the shadow length that the frozen-dried cells have retained a three-dimensional form, while the air-dried specimen is extremely flattened.

Figs. 2 *a* and 2 *b* are electron micrographs of frozen- and air-dried marginal bands isolated from erythrocytes of the lizard *Dipsosaurus dorsalis*. The elements of the bands, which are intimately associated with membranous material, appear in the frozen-dried preparation as smooth cylindrical bodies, with diameters of 400 to 500 A. In the air-dried control, the band elements are collapsed, and have diameters of 200 to 250 A. The role of marginal bands and a comparison of their morphology after different preparative techniques have been discussed elsewhere (6–9).

Thermoelectric cooling devices provide an easy, inexpensive method of preparing as routine frozen-dried specimens for electron microscopy. The cost of the Ohio Semiconductor module and power supply was approximately $80. The primary disadvantage in the method we have described is the relatively high temperatures attained by the coolers. Temperatures about −30°C are too high to provide very fast freezing and to preclude the extensive formation of ice crystals (10, 11); as a result, the devices as we have used them are applicable to a limited variety of specimens. Our method appears to preserve three-dimensional structure in these specimens, and preliminary evidence indicates that it will be useful for particles such as bacteriophage and ribosomes.

Several manufacturers have more recently produced thermoelectric freezers which may be capable of lower temperatures (some distributors are Central Scientific Co., Chicago; Preiser Scientific, Inc., Charleston, West Virginia; Melcor, Trenton, New Jersey). It should be pointed out that although the temperature gradients produced by these devices are large, the amount of heat they can pump is very low, so that large specimens can not be rapidly frozen. The capacity of the freezers can be increased by using several units in series, but relatively small decreases in temperature are achieved in this way.

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